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High Incidence of Antinuclear Antibodies That Recognize the Matrix Attachment Region

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The matrix attachment region (MAR) is a distinctive genomic DNA involved in a variety of nuclear processes through association with the nuclear matrix. Recent studies suggest that nuclear matrix is altered in the process of apoptosis and presented to the immune system, leading to the production of autoantibodies against its protein components. To see whether MARs are also recognized by autoantibodies, a collection of human sera containing antinuclear antibodies was screened for the presence of binding activities against cloned MARs. We found that MAR-binding activities are quite common in these sera. There was a positive correlation among the MAR-binding titers for three different MAR probes. As expected, the MAR-binding activity was copurified with serum IgG, and subclass analysis with affinity-purified IgG on MAR-Sepharose showed a predominance of IgG2 isotype. Several lines of evidence implied that the anti-MAR antibodies detected here is distinct from the ordinary anti-DNA antibodies that are reactive to bulk DNA.

Key Words: **nuclear matrix; matrix attachment region; apoptosis; autoimmune disease; autoantibodies; antinuclear antibodies; anti-DNA antibodies.**

The nuclear matrix is a proteinaceous network structure in the interphase nucleus that is believed to be involved in many nuclear processes like DNA replication, transcription, and RNA processing (1). Genomic DNA is organized into topologically independent looped domains by specific interaction with nuclear matrix through a noncoding DNA sequence termed collectively as the matrix attachment region (MAR). Although no distinctive sequence motifs are discernible in MARs, they do share a significant feature that is a base composition with high AT content. Several nuclear matrix proteins that specifically bind MARs have been identified (2–4). These proteins are thought to be responsible for the site-specific anchorage of genomic DNA and thus important for various nuclear functions (5).

Frequent occurrence of antinuclear (auto)antibodies (ANAs) has been noted in some disease groups with autoimmune response, most frequently in systemic lupus erythematosus (SLE), rheumatoid arthritis, and mixed connective tissue disease. ANAs produced in autoimmune patients are usually a mixture of antibodies that react either with distinctive nuclear proteins, RNA–protein complexes like the small nuclear ribonucleoprotein (snRNP), DNA–protein complexes (nucleosome), or isolated DNA (6). The spectrum of autoantigens recognized by ANAs differs significantly in individual patients, but it has a certain similarity within the same disease categories. Some ANAs are referred to as "antinuclear matrix antibodies" because they show a strong immunostaining of nuclear matrix preparations after indicator cells were treated with detergent, high salt, and DNase (7). Several nuclear matrix proteins have been identified as a specific autoantigen by immunoblotting procedures (8).

Anti-DNA autoantibodies are generally classified into two groups, anti-ssDNA and anti-dsDNA antibodies, by comparing relative affinities to single-stranded and double-stranded DNA molecules, respectively. It has been reported that sera from autoimmune patients, mostly SLE, contain autoantibodies that can recognize more specific structural features such as Z-DNA (9), palindromic sequences (10), triplex or quadruplex DNA (11, 12), telomeric repeats (13), and other specific sequences (14). These studies, together with the frequent occurrence of antinuclear matrix autoantibodies, imply the presence of autoantibodies with significant affinity to MARs. We show here for the first time that anti-MAR antibodies are indeed frequently

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detected in ANA-positive sera, suggesting a probably involvement of MARs in the autoimmune response.

MATERIALS AND METHODS

Serum samples. Randomly chosen patient sera for clinical tests were screened for ANA by immunofluorescence staining of HEp-2 cells (see under "immunofluorescence microscopy") and total of 136 ANA-positive sera were selected. These sera showed either diffuse, peripheral, punctate or speckled nuclear staining patterns. As a control, 30 serum samples were collected from healthy volunteers.

MAR plasmids. Three plasmids (pBRCEN3, pFTZ, and pAR1) that contained inserts from the centromeric region of *S. cerevisiae* chromosome III (15), the upstream element of the *Drosophila* fushitarazu gene (16), and the intronic sequence of mouse Igκ gene (17), respectively, were used. The plasmids were digested either with *Bam*HI/*Eco*RI (pBRCEN3), *Eco*RI (pFTZ), or *Bam*HI/*Hin*dIII (pAR1) and subjected to electrophoresis in 1% agarose gels. MAR-containing fragments of 1 kb (pBRCEN3), 1.2 kb (pFTZ), and 0.37 kb (pAR1) were recovered from the gel, purified, and ^{32}P-labeled on both ends in the end-filling reaction with Klenow polymerase as described previously (4).

MAR binding assay. One microliter of serum samples or diluted IgG fractions were incubated at 30°C for 20 min with ^{32}P-labeled MAR probes (10^4–10^5 cpm) and 0.25 mg/ml unlabeled *E. coli* DNA (sonicated and nuclease S1-treated) in a binding buffer containing 0.1 M Tris–HCl (pH 7.5), 0.25 M NaCl, and 2.5 mM EDTA (final volume = 0.1 ml). *E. coli* DNA does not contain MAR and serves as a nonspecific competitor. The mixture was passed through a prewetted nitrocellulose filter held in a slot blotter (Bio-Dot SF, Bio-Rad Labs) and the blotted filter was washed with 0.4 ml of the binding buffer under negative pressure, followed by additional washing in a plastic container. After exposing the dried filter against X-ray film for autoradiography, the radioactivity of blotted area cut out from the filter was measured by scintillation counting. Relative MAR-binding activities in test sera were expressed by percentages of the input radioactivity retained on filter. ANA-positive sera that showed a high binding to all MARs were selected for further analysis (designated by sample numbers 13, 19, 21, and 27 in the text).

Anti-DNA antibody assays. Titers for anti-dsDNA and anti-ssDNA antibodies were determined according to the published methods (18, 19). Calf thymus DNA (Sigma) was used as an antigen for microplate ELISA and allowed to react with 200-fold-diluted human serum. Alkaline phosphatase-conjugated affinity purified $F(ab')_2$ goat anti-human IgG antibody (EY Labs) was used as the secondary antibody and the substrate was *p*-nitrophenyl phosphate (Sigma). The absorbance at 405 nm was measured in duplicates on a photometer (Bio-Rad Labs Model 450). Each titer (IU/ml) was obtained from the standard curve using the known standard antibody (ANA standard serum, MBL, Nagoya, Japan).

Analysis of anti-MAR antibodies. pFTZ was digested with *Eco*RI and the 1.2-kb MAR fragment and 3-kb vector fragment were purified by agarose gel electrophoresis. These DNA fragments were immobilized on Sepharose beads (CNBr-activated Sepharose 4B, Pharmacia) to prepare MAR beads and vector beads. Serum samples containing 1 mg of IgG were first mixed with the vector beads (0.2 ml) in the binding buffer consisting of 10 mM Tris–HCl (pH 7.5), 0.3 M NaCl, 1 mM EDTA, and 0.5% Empigen BB, a detergent. After incubating for 1 h at 30°C, vector beads were removed by centrifugation and supernatants were incubated with MAR beads under the same conditions. The beads were washed with the binding buffer and then incubated with mouse monoclonal antibodies that are specific to human IgG subclasses 1–4 (Zymed) in separate tubes (100 ng protein per tube). Washed beads were resuspended in SDS–PAGE sample buffer, boiled for 2 min, and subjected to electrophoresis in 10% polyacrylamide gels. After transfer onto nitrocellulose membranes, mouse antibodies (all IgG1) were detected by an immunoblotting procedure with peroxidase-labeled anti-mouse immunoglobulins (KPL).

Immunofluorescence microscopy. HEp-2 cells fixed on microscope slides (Fluoro HEPANA Test, MBL) were incubated with human sera, diluted to 20- to 100-fold in PBS containing 1% BSA, for 30 min at 37°C in a moist chamber. After washing the slides in PBS, cells were incubated with FITC-labeled goat antibody against human immunoglobulins (KPL) as described above and observed under a fluorescence microscope. In some experiments, nuclear matrix was prepared *in situ* according to the published procedure (20) and immunostained as above. Briefly, HeLa cells grown on slides were first extracted with a detergent mixture in the presence of RNase inhibitor, vanadyl ribonucleoside complexes, and treated with RNase-free DNase I. Chromatin proteins were then extracted with 0.25 M ammonium sulfate and the resulting nuclear matrix was fixed with 4% paraformaldehyde in PBS.

Other methods. IgG was purified from serum samples using a protein G affinity purification kit (MAbTrap GII, Pharmacia). Serum IgG levels were measured from the absorbance at 700 nm by the immunoturbidity method (N-assay TIA IgG-S, Nittobo, Japan) using an automatic analyzer (Hitachi 7070) (19). Statistical analyses of the data were performed by the computer software StatView. Statistical significance and correlation analyses were done by Mann-Whitney U test and Spearman rank correlation test, respectively.

RESULTS

ANA-positive sera were first selected by immunofluorescence screening of patient sera on HEp-2 cells and their MAR-binding activities were compared with those of ANA-negative control sera from healthy subjects. Diluted serum samples were subjected to nitrocellulose filter-binding assay with three different MAR probes labeled with ^{32}P that were originated from the centromeric region of *S. cerevisiae* chromosome III (Cen III), the upstream element of the *Drosophila* fushitarazu gene (Ftz), and the intronic sequence of mouse Igκ gene (Igκ). Nonspecific interactions between the probes and serum proteins were suppressed by a large excess of unlabeled *E. coli* DNA in a high ionic strength buffer (0.25 M NaCl). As shown in Fig. 1, comparison of the relative radioactivities retained on filter revealed that the levels of MAR-binding activity in the ANA-positive group are significantly higher than those of the control group for all MAR probes ($P < 0.001$). The results thus suggest the enrichment of a proteinaceous entity in ANA-positive sera, most likely to be immunoglobulins, that selectively binds to MARs. Since the concentration of IgG in ANA-positive sera was considerably higher than that in control group ($P < 0.0001$), the observed difference in the MAR-binding activity might be simply due to the elevated IgG level. However, this possibility was ruled out by the fact that the IgG content showed only a weak positive correlation to the binding of Cen III or Ftz but no correlation to that of Igκ MAR (data not shown).

MARs are different each other in nucleotide sequence but they share a common character, the selective binding to the nuclear matrix. To see whether

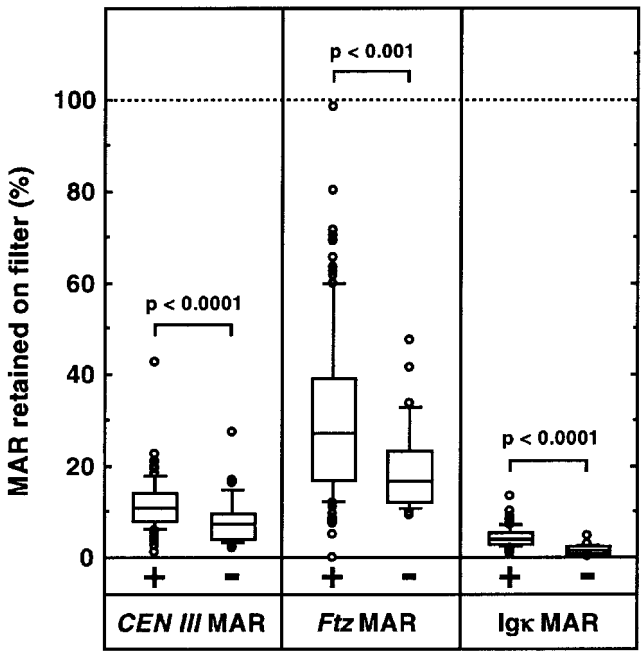

FIG. 1. MAR-binding activities of serum samples measured by a nitrocellulose filter-binding assay with different MAR probes. Levels of binding activities between the two groups of sera, ANA-positive (+) and ANA-negative (−), were compared by using the StatView program. Box plots are shown, where each box contains 80% of data points and horizontal lines inside the box designate the median value. *P* values obtained from the Mann–Whitney *U* test are also shown.

similar situation applies to the MAR-binding activity in ANA-positive sera, correlational analysis was performed between all three combinations of MARs (Fig. 2). As expected, positive correlations were found between the MARs in the ANA-positive group ($P < 0.0001$), whereas no significant correlations were detected in ANA-negative sera (not shown). Since the anti-ssDNA titer was significantly higher in the ANA-positive group ($P < 0.01$), the MAR-binding activity might be related to the titer of anti-DNA antibodies. The binding activity for all MARs, however, showed no positive correlations to either anti-ssDNA or anti-dsDNA titers (not shown). These results suggest that the MAR-binding protein in ANA-positive sera is capable of recognizing a structural feature shared by MARs and is distinct from the anti-DNA antibodies that are detectable with bulk DNA antigen.

To clarify the MAR-binding entity in ANA-positive sera, IgG was purified from a selected serum with high MAR-binding activities and subjected to the filter-binding assay with increasing concentrations of IgG (Fig. 3). The result showed that the protein factor responsible for the binding of MARs is indeed copurified with IgG. As predominance of a particular IgG subclass is notable in some autoantibodies (6), we next determined the subclass of anti-MAR antibodies after an affinity purification step on immobilized MAR fragments (see Materials and Methods). The MAR-binding antibodies bound to the Ftz MAR-Sepharose were incubated with subclass-specific monoclonal antibodies and the bound mouse antibodies were detected by immunoblotting with anti-mouse immunoglobulin antibody labeled with peroxidase (Fig. 4). Under these conditions, the antibody bound to the beads was mainly anti-IgG2, which was more abundant with the ANA-positive serum. Similar results were obtained with three other ANA-positive sera possessing high anti-MAR titers (not shown), suggesting that IgG2 is the predominant isotype of anti-MAR autoantibodies.

We finally assessed the subnuclear distribution of autoantigens recognized by ANA-positive sera with high anti-MAR titers (Fig. 5). When HEp-2 cells were stained, both ANA-positive sera showed a nuclear staining characterized by multiple spots. Similar pattern was observed with HeLa cell nuclear matrix preparation (Fig. 5A). Since this punctate distribution of autoantigen resembles that of centromeric regions in interphase nuclei, the molecular target of these sera was analyzed by immunoblotting using a control serum containing autoantibodies to CENP-B, a major centromere protein (Fig. 5B). As expected, both sera recognized a 80 kDa-protein band that comigrated with CENP-B. The persistent staining of nuclear matrix is consistent with the report that interphase centromeres or prekinetochores are associated with nuclear matrix (21). Immunofluorescence microscopy with other anti-MAR sera (#13 and #27) revealed a fibro-granular nuclear staining pattern with corona-like perinuclear stain (not shown). These data indicate that in addition to anti-MAR antibodies ANA-positive sera with high anti-MAR titers also contain antibodies to nuclear pro-

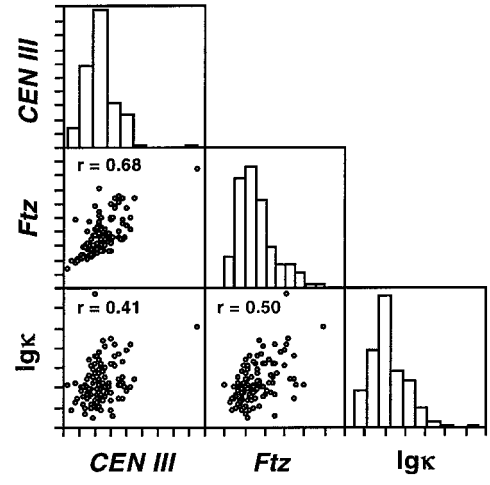

FIG. 2. Correlations between the MAR-binding activities for three pairs of MAR combination. MAR-binding activities of ANA-positive sera were plotted on arbitrary scales. Correlation coefficients (*r*) were calculated by the Spearman rank correlation test. Frequency distributions of MAR-binding activity for each probe are also shown as histograms.

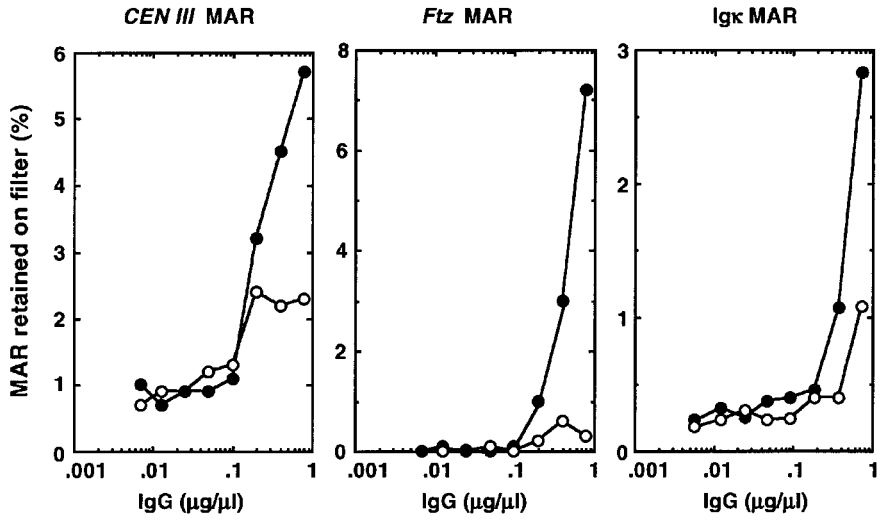

FIG. 3. Binding of MARs with purified IgG. MAR-binding activities were measured by the filter-binding assay with IgG preparations from the ANA-positive serum No. 19 (closed circles) and an ANA-negative control serum (open circles).

teins, including nuclear matrix proteins. There was no direct correlation between the anti-MAR titers and the patterns of autoantigen distribution. It appeared, however, that the most frequent staining patterns are those mentioned above, nuclear spots and fibrogranular staining, when a larger set of anti-MAR sera was examined.

DISCUSSION

How is the production of anti-MAR antibodies initiated? Recent studies argue that a major population of ANAs in SLE is anti-nucleosome antibodies (22). Nucleosomes are released from chromatin by endonuclease attacks in the process of apoptosis. There is a growing evidence that in SLE apoptosis is disturbed or clearance of apoptotic cells is impaired, leading to an increased presentation of nucleosomes as autoantigen on cell surface that drives a T-cell-dependent autoimmune response (23, 24). It is worth considering here that during apoptosis structural and compositional changes in the nuclear matrix have been reported (25). Notably, a number of nuclear matrix proteins undergo proteolytic fragmentation in an early stage of apoptosis. These include the nuclear lamina protein lamins A/B (26), poly(ADP-ribose) polymerase (27), the nuclear mitotic apparatus protein NuMA (28), a tissue-specific MAR-binding protein SATB1 (29), the 70-kDa protein of U1 snRNP (30), and hnRNP U protein (31). The hnRNP U protein, also called SAF-A or SP120, is a MAR-specific DNA-binding protein and believed to be involved in the anchorage of genomic DNA (3–5). The consequence of its fragmentation is two-fold, because the caspase-dependent cleavage in its MAR-binding domain results in disruption of the looped domain organization of chromatin, facilitating subsequent nuclear condensation and generation of oligonucleosome-sized DNA ladders (31). Interestingly, most of the proteins that are cleaved during apoptosis are also the target of autoantibodies (30). Based on these observations, we speculate that the altered structure of nuclear matrix in apoptotic cells renders the nuclear matrix components, including MARs, more accessible to the immune system to induce the production of anti-MAR antibodies.

It has been proposed that anti-DNA antibodies are initially raised against protein epitopes since DNA *per se* is a poor immunogen and some anti-DNA antibodies are cross-reactive to cellular proteins (32, 33). Recent studies also suggest the importance of DNA-protein complexes for the production of anti-DNA antibodies through a hapten-carrier like scheme, leading to activation of antigen-specific helper T cell clones (34–36). Many proteins involved here are likely to be DNA-

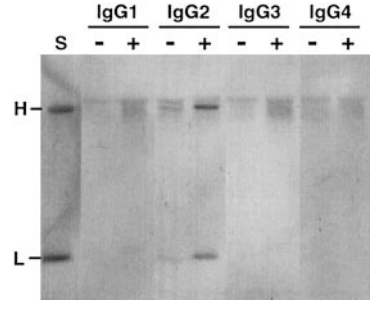

FIG. 4. Subclass assignment of the anti-MAR antibody. Antibodies reactive to Ftz MAR were affinity-purified from the ANA-positive serum No. 19 (+) or ANA-negative control serum (−), and allowed to react with mouse monoclonal antibodies specific to human IgG isotypes (indicated on top). Mouse antibodies were then detected by immunoblotting with a mouse IgG standard (lane S). Heavy (H) and light (L) chains of IgG are indicated on the left.

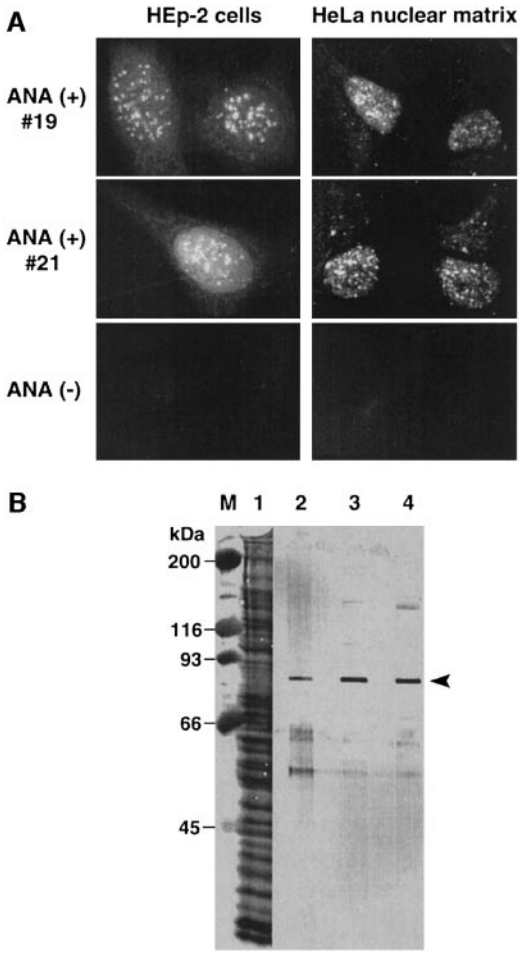

FIG. 5. Analysis of autoantibodies reactive to nuclear proteins in ANA-positive sera with high anti-MAR titers. A, immunofluorescence micrograph; B, immunoblotting with total nuclear proteins. Lane M, molecular weight marker; lane 1, protein staining; lane 2, anti-centromere autoimmune serum; lane 3, ANA-positive No. 19 serum; lane 4, ANA-positive No. 21 serum. The arrowhead designates the centromere protein, CENP-B.

binding proteins. An emerging concept is that these proteins are probably functional components of a large protein complex and epitopes recognized by autoantibodies constitute their active sites or functional domains (37). It is possible, therefore, that anti-MAR antibodies are cross-reactive to the DNA-binding domain of a MAR-binding protein. Alternatively, anti-MAR antibodies could be anti-idiotype antibodies that are driven against the antibodies to MAR-binding domains. We have not investigated these possibilities, including the coincidence of anti-CENP-B and anti-MAR antibodies (Fig. 5), but they are certainly intriguing possibilities to pursue.

In the present study MAR binding assay was conducted in the presence of competitor DNA, and the IgG reactive to MAR-Sepharose was not removed by the preadsorption step with vector DNA-Sepharose. These data, together with the lack of correlation between the titers for anti-MAR and anti-DNA antibodies, indicate that the binding specificity of anti-MAR antibodies must be significantly different from that of prevalent anti-DNA antibodies. An important sequence motif in MARs that may interact with MAR-specific DNA-binding proteins is thought to be the short AT-tract which is abundant in AT-rich MARs (38, 39). This is simply demonstrated by the strong competitive binding of a synthetic DNA, poly(dA) · poly(dT). MAR-binding proteins are likely to recognize the minor groove of AT-tracts which is narrower than that of average B form DNA. Anti-MAR antibodies may discriminate MARs from bulk DNA by a similar mechanism since the binding of anti-MAR antibodies was also susceptible to poly(dA) · poly(dT) and there was a positive correlation among the anti-MAR titers for different MARs.

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